Mycoremediation of Sewage Treatment Plant water by Mycofiltration using fungal mycelia

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INTRODUCTION

In developing countries like India, the problems associated with wastewater reuse arise from its lack of treatment. The challenge thus is to find such low-cost, low-tech, user friendly methods, which on one hand avoid threatening our substantial wastewater dependent livelihoods and on the other hand protect degradation of our valuable natural resources. Mycoremediation term define the phenomenon of fungi using for remediation purposes. Fungal mediated bioremediation generally uses extracellular enzymes to breakdown the pollutants and reduce or remove the toxic waste from the environment(Kulshreshtha *et al.*, 2010). Fungi mycelium can also works as a filter network used in buffer zones around streams to filter the run-off from farms, highways and suburban zones(Chiu *et al.*, 2000). In other words it is a form of bioremediation, the process of using fungi to degrade or sequester contaminants in the environment. Stimulating microbial and enzyme activity, mycelium reduces toxins in-situ. Some fungi are hyperaccumulators, capable of absorbing and concentrating heavy metals in the mushroom fruit bodies(Ozcan *et al.*, 2013).

As we know that due to large availability of STP treated wastewater for irrigation purposes, the chances of toxic compounds accumulation in plants and edible crops also increases due to biomagnifications phenomenon it already affecting human's health. Generally STP treated wastewater considered as a good source of nutrient for plant growth but it also have toxic ions and harmful microbial load. To remediate these harmful substances and microbes before irrigation process without affecting its nutritional value, some safe and low cost treatment method prior to irrigation is required. There have been a number of risk factors identified for using reused waters for purposes such as agricultural irrigation. Some risk factors are short term and vary in severity depending on the potential for human, animal or environmental contact (eg, microbial pathogens), while others have longer term impacts which increase with continued use of recycled water (eg, saline effects on soil). The most common human microbial pathogens found in recycled water are enteric in origin. Enteric pathogens enter the environment in the faeces of infected hosts and can enter water either directly through defecation into water, contamination with sewage effluent or from run-off from soil and other land surfaces(Mara and Feachem, 1995).

The types of enteric pathogens that can be found in water include viruses, bacteria, protozoa and helminths. The risk of water-borne infection from any of these

pathogens can be reliant on a range of factors including pathogen numbers and dispersion in water, the infective dose required and the susceptibility of an exposed population, the chance of faecal contamination of the water and amount of treatment undertaken before potential exposure to the water.

Bacteria are the most common of the microbial pathogens found in recycled waters(Toze, 1999). There are a wide range of bacterial pathogens and opportunistic pathogens which can be detected in wastewaters. Many of the bacterial pathogens are enteric in origin, however, bacterial pathogens which cause non-enteric illnesses (e.g., *Legionella* spp., *Mycobacterium* spp., and *Leptospira*) have also been detected in wastewaters(Toze, 2006). Bacterialpathogens are metabolically active microorganisms that are capable of self-replication and are therefore, theoretically capable of replicating in the environment. In reality, however, these introduced pathogens are prevented from doing so by environmental pressures(Gordon and Toze, 2003). Like other enteric pathogens, a common mode of transmission is via contaminated water and food and by direct person to person contact(Maciel and Sabogal-Paz, 2016). A number of these bacterial pathogens can also infect, or be carried by wild and domestic animals.

The method which is suitable for remediating toxic pollutants and bacterial loads from STP water is Mycofiltration. It is a technique in which mushroom forming fungi purposely grow on substrates as a biologically active filtration media. The use of intentionally vegetative network of mycelium to facilitate water quality improvements in engineered ecosystem. The concept of mycofiltration first introduced by Paul Stamets in 1993; According to Paul Stamets, the future of mycoremediation lies in Mycological Response Teams. These teams would consist of knowledgeable and trained people who would set up centers that would use mycoremediation techniques to recycle and rebuild healthy soil in the area. This begins by spreading awareness and information in regards to the benefits of mycoremediation.

Mushrooms are not plants, and require different conditions for optimal growth. All mushroom growing techniques require the correct combination of humidity, temperature, substrate (growth medium) and inoculum (spawn or starter culture)(Subbiah and Balan, 2015). Wild harvests, outdoor log inoculation and indoor trays all provide these elements. Fungiculture is the process of producing food, medicine, and other products by the cultivation of mushrooms and other fungi.Mycelium, or actively growing mushroom culture,

is placed on a substrate—usually sterilized grains such as rye or millet—and induced to grow into those grains(Chiu *et al.*, 2000). This is called inoculation. Inoculated grains are referred to as spawn. Spores are another inoculation option, but are less developed than established mycelium. Since they are also contaminated easily, they are only manipulated in laboratory conditions with a laminar flow cabinet.Instead of seeds, mushrooms reproduce asexually through spores. Spores can be contaminated with airborne microorganisms, which will interfere with mushroom growth and prevent a healthy crop(Dressaire *et al.*, 2016; Hassett *et al.*, 2015).

Mushrooms are of many types approximately 14,000 of mushroom species are described. *Ganoderma lucidum, Calocybe indica* and *Pleurotus ostreatus* belong to three different families, classification of these three mushroom species are given below (table no. 1)(Rzymski *et al.*, 2016; Subbiah and Balan, 2015). For effective mycofiltration combination of different mushroom species are required, on the basis of high remediation capabilities. Generally fungi and bacteria are used for remediation purposes but both microbes kill each other for the breakdown of pollutants or inhibit each other growth. Here we screen two types of mushroom (red and white) on the basis of antimicrobial activity against harmful bacteria present in STP water of dayalbagh.

Table 1: Classification of selected mushrooms species

Ganodermalucidum	***Calocybeindica***	***Pleurotusostreatus***
Kingdom: Fungi	Kingdom: Fungi	Kingdom: Fungi
Phylum: Basidiomycota	Phylum: Basidiomycota	Phylum: Basidiomycota
Class: Agaricomycetes	Class: Agaricomycetes	Class: Agaricomycetes
Order: Polyporales	Order: Agaricales	Order: Agaricales
Family: Ganodermataceae	Family: Lyophyllaceae	Family: Pleurotaceae
Genus: *Ganoderma*	Genus: *Calocybe*	Genus: *Pleurotus*
Species: *G. lucidum*	Species: *C.indica*	Species: *P.ostreatus*

OBJECTIVES

Present investigation entitled "**Mycoremediation of Sewage Treatment Plant water by Mycofiltration using fungal mycelia"** consists of following objectives given below:

1. Analysis of general physico-chemical parameters including heavy-metals & bacteriological assay of STP site in Dayalbagh

2. Fungal spawn preparation and it's cultivation for the making of mycofilters to remediate heavy metals and pathogenic bacteria

3. Screening of cultivated mushroom species through antibacterial activity test against pathogenic bacteria procured from MTCC

4. To check the heavy metals and pathogenic bacteria remediation through mycofiltration technique

REVIEW OF LITERATURE

Like most Indian cities, Agra metropolis is growing. The municipality, encompassing an area of 121.57 sq km had a population, as per 2001 census, of about 1.26 million. By 2011, this had grown to 1.6 million, with 2018 projected population estimated to touch 2.6 million. But Agra, designated as a world heritage site, faces a number of challenges in terms of water, sewerage and financing municipal works. There is a bursting strain on the infrastructure and services, both from its own population and from the regular onslaught of visiting tourists, estimated at 1.80 million every year. In the city, Agra Jal Sansthan (AJS) is in charge of operation and maintenance, and revenue collection in supplying water, while all capital works related to water supply and sanitation are undertaken by Agra Jal Nigam (AJN).

According to the AJS, the total water demand of the city is 320 million litres per day (mld), which includes the demand for bulk supply, estimated at 75 mld. The water demand as estimated for the 1.42 million-population in 2005 was 245 mld, which was calculated on a 170 litres per capita daily (lpcd) standard. For this, the city has two water treatment plants with a capacity to treat 410 mld in entirety. The NRCD monitors the river at two places in Agra — upstream at Poiaghat, (182 km from Okhla barrage towards Dayalbagh) and downstream behind the Taj Mahal 192 km from the Okhla barrage.

According to CPCB's *Status of sewage treatment in India* report of February 2006, the city generated 211 mld sewage in 2001. This is based on a sewage generation factor of 168 lpcd (or a210 lpcd water supply). UPJN estimates show that the water demand has shot up from 284 mld to 320 mld leading to an increased wastewater discharge. But how much is actually used is unknown. UPJN while reviewing YAP has estimated the wastewater discharge in 2003 to be 152.15 mld. This assumes the water supply to be 107 lpcd. This is far lower that the water supply estimates provided by AJS. R P S Sanghu, chief chemist AJS says, "Per capita water supply is set at 135 lpcd." This difference in data will definitely affect the waste planning for the city. The most recent estimates, however, have been collated by CPCB in its 2005-06 annual report stating the flow in all drains to be 254 mld. This points to a 100 mld rise in waste water generated over since UPJN's last estimate 3 years back.

Three STPs with a combined capacity of 90.25 mld have been set-up under YAP. While 78 mld (Dhandupura) and 2.25 mld (Burhi ka nagla) facilities were set-up to deal with waste of Cis Yamuna, a solitary 10 mld STP was set-up at PeelaKhar to deal with

the waste of trans-yamuna. The sewage network has been expanded to feed them, but the infrastructure remains inadequate and the river remains dirty. Agra's sewerage system is in shambles. Spread over 1,400 ha it is devoid of proper connections with most sewage flowing into open drains. While the system is largely silted several lines remain choked and damaged at a number of places. This has made the disposal of sewage into nalas (open drains) a common affair. For example, the sewage arriving at Dhandupura STP is partly from the small population connected to the sewerage system, with the rest arriving from the 17 intercepted open drains. On the other hand, incoming sewage at BurhikaNagla and PeelaKhar STP arrives from intercepted open drains. Notably, a major part of Agra, 8,300 ha remains unsewered.

Mycoremediation has held promise for cleaning up polluted soils since 1985 when it was discovered that white rot fungus *Phanerochaete chrysosporium* could metabolize a number of important environmental pollutants, including polychlorinated biphenyls (PCB's), chlorophenols, dioxins, DDT, trinitrotoluene, polycylic aromatic hydrocarbons (PAH's) and synthetic dyes(García-Delgado *et al.*, 2015) (Helmfrid *et al.*, 2012). This ability to degrade chemical pollutants of different compounds is caused by the extracellular enzyme system (lignin peroxidase, manganese-dependent peroxidase and laccase) and production of free radicals(Fernández-Fueyo *et al.*, 2014; da Luz *et al.*, 2012; Salame *et al.*, 2013). Mycoremediation is a type of bioremediation that utilizes fungal enzymes' natural ability to break down anthropogenic contaminants, allowing ecological succession to take place naturally. Succession is the gradual replacement of one group of organisms by another over time following initial disturbance.

Soil contaminated by PAHs is well documented; the persistence and compounding of PAHs are tantamount to significant ecological risk because of toxicity, potential carcinogenicity, and resistance to bioremediation. Therefore, these compounds are on the US EPA's Priority Pollutant list, which includes 129 different pollutants. Using experiments with fungi and yeasts naturally found in aquatic sediment, surface waters, and terrestrial environments, Cabello (2001) found that fungi and yeasts can be used to biodegrade PAHs. Fungi can penetrate the soil whereas bacteria cannot; fungi accomplish this penetration by sending out infiltrating fungal hyphae that exudeenzymes which reach subsoil PAHs. Other lignin-degrading fungi enzymes candecompose wood and break down and degrade a variety of toxins (Cabello, 2001). Eggen and Sasek (2002) describe the capacity of spent substrate from commercial mushroom production of *Pleurotus ostreatus* to

remove PAHs from weathered creosote inhighly contaminated soil from an abandoned wood preservation site. Adding the spentfungal compost resulted in reductions from 50% (acenaphthene, anthracene) to 87% (phenanthrene, flourene) within a twelve-week treatment. The reduction increased to 87% (anthracene) and 97-99% (phenanthrene, flourene, acenaphthene) after additional reinoculation with fungal substrate and an added three-week incubation period. After atwelve-week fungal treatment period flouranthene and pyrene decreased by approximately 43% and 34%, respectively. Spent compost of *Agaricus bisporus*, commonly known as white buttonmushroom, has been utilized by gardeners to fertilize and condition soils on disturbed commercial sites. Several species of fungi (*Pleurotus ostreatus, Trametes versicolor, Agrocybeaegerita, Kuehneromyces mutabilis* and *Stropharia rugosoannulata*), are edible and medicinal, commonly used to benefit human health, and have been extensively studied (Eggen et al. 2002). These also enable environmental health by metabolizing chemical toxins.

The search for low-cost methods for biodegradation of pollutants justifies favouring the use of the whole microorganism or unpurified culture broth. As demonstrated by several studies, mushroom ligninolytic enzymes can degrade the pollutants that otherwise would be difficult to eliminate from the environment. The application of the pure, isolated enzymes or of mixtures with defined composition ensures specific reactions of substrates without side-products being formed(El Enshasy and Hatti-Kaul, 2013; Melgar *et al.*, 2014). The successful implementation of the mycoremediation to the technical scale involves, according to Singh, 2013 the development of three phases of the process: 1) the inoculum preparation techniques and their improvements, 2) the clear technical protocols for the final design and associated engineering processes, 3) the remediation protocols for the monitoring, adjustment, continuity, and maintenance of the engineering system.

The significant part of biotechnological processes described in the work is still is at the stage of preliminary experiments. However, the processes using white-rot fungi for degradation of the environmental pollutants have been patented. Recently a few companies, including Gebruder Huber Bodenrecycling in Germany and EarthFax Development Corporation inUnited States employ white rot fungal cultures for soil bioremediation and a broader use probably will take place in the future. Myco-facilitation can help to transform a degraded location into a thriving ecosystem with increased diversity. Therefore, mycoremediation needs well trained personnel with sufficient knowledge of microbiology.

Both intrinsic (deeper study of the influence of mycoremediation process) and extrinsic (new possibilities for fungal species) research is needed to bring about the desired results.

(Pinedo-Rivilla *et al.*, 2009; Tigini *et al.*, 2009) find approximately 300 references covering the period 2005-2008, describes the use of fungi as a method of bioremediation to clean up environmental pollutants.

(Liao *et al.*, 2012) suggests that *G. lucidum* biomass could be used for treating water containing zirconium ions. *Ganoderma lucidum* to remove Zr(IV) ions from aqueous solutions in batch system. The biosorption characteristics of *G. lucidum* for Zr(IV) ions were evaluated as a function of medium pH, biomass dosage, contact time, initial zirconium concentration and temperature. Maximum zirconium uptake (142.5 mg/g) was observed at pH 3.5.

(Ejikeme and Henrietta, 2010) suggest that *P. squarrosulus* possessed broad-spectrum of activity against microbial isolates used. The significance of antimicrobial activity of mushroom extracts was compared with the standard antibiotics (gentamicin, 5 µg/disc) using chi –square. There were significant difference between the mean zone of inhibition of the ethanol extract of *P. squarrosulus* and the standard antibiotic against test organisms at 5% level.

(Osman *et al.*, 2011) evaluated the antibacterial and antifungal activity of aqueous extract of mycelia from five mushroom strains on selected bacterial pathogens. Mycelial extracts from submerged cultures of mushrooms showed potential antimicrobial activities against the selected bacterial and fungal strains.Tested mushrooms showed higher levels of endopolysaccharides (IPS) than exopolysaccharides (EPS).

(Avcı *et al.*, 2014) work was the determination of antioxidant activities of the ethanol extract from three mushrooms (*Ganodermalucidium, Lentinulaedodes and Coprinusmicaceus)* by using DPPH radical scavenging and total antioxidant status assays. All mushrooms used in this study were found to have antimicrobial effects at a variety of degrees against microorganisms tested.

(Lindequist *et al.*, 2005; Sanodiya *et al.*, 2009) describes pharmacologically active compounds from mushrooms. **(Sanodiya *et al.*, 2009)** focuses on the pharmacological aspects, cultivation methods and bioactive metabolites playing a significant role in various therapeutic applications.

(Purnomo *et al.*, 2010) results indicate that SMW from P. ostreatus is a medium which can be potentially used for bioremediation in DDT-contaminated environments.The SMW from P. ostreatus degraded the DDT by 40% and 80% during 28 d incubation in sterilized (SL) and un-sterilized (USL) soils,respectively. [U-14C]DDT was mineralized by 5.1% and 8.0% during 56 d incubation in SL and USL soils, respectively.

(Adedokun, 2015) A simulation experiment was carried out in which oyster mushrooms (*Pleurotus pulmonarius*) was grown on used engine oil.The mycelia of the mushroom were used to inoculate Spent Engine Oil (SEO, 10% (v/w)) polluted soil. After four weeks of incubation, fruiting bodies growing on the polluted soil were analyzed for remnant hydrocarbon profile. Results showed that total PolycyclicAromatic Hydrocarbon (PAH) was 10 mg/kg and Aliphatic Hydrocarbon (AH) was 23 mg/kg.

(Gupta *et al.*, 2010) Study shows the potential of various fungi for their capacity to decolorizethe textile effluent and their capability for heavy metal removal. Maximum decolorization (92%) was achieved by *Trametes versicolor*, whereas maximum concentration of chromium was removed by *Mucor hiemalis*.

PROPOSED METHODOLOGY

Proposed work will be completed by following methods:

1. Sample collection from Sewage treatment plant water in Dayalbagh for analysis of general physico-chemical parameters including heavy-metals & bacteriological assay

- Physicochemical parameters of STP water will be determined by APHA protocols; Effluent samples were analyzed for physicochemical parameters such as pH, temperature, salinity, conductivity, BOD (Biological Oxygen Demand) and total dissolved solids (TDS) by water analyzer kit. Chemical Oxygen demand (COD) is done by the method as given in American Public Health Association (APHA) manual (1995).
- Heavy metals determination will be done using two methods for comparison and qualityassurance purposes. The methods will be used atomic absorption spectrometer (Varian–AAS) and Gas-Chromatography Mass Spectroscopy (GC-MS) respectively. Sampledigestion will be carried out according to METHOD3050B of the USEPA: Acid Digestion ofSediments (USEPA, 2012).
- All these parameters will be analyzed within 24 hrs. in each of the three replicates.
- Bacteriological assay will be determined

2. Mother culture of mushroom species will be available from Solan (H.P) **National Research Centre of Mushroom *(Indian Council for Agricultural Research)* Chambagaht, Solan – (HP) 173213 India**

- *Ganoderma lucidum* (Reishi Mushroom) (Bidegain *et al.*, 2015; Klupp *et al.*, 2015; Mesa Ospina *et al.*, 2015; Rani *et al.*, 2015; Rzymski *et al.*, 2016; Saylam Kurtipek *et al.*, 2016)
- *Calocybe indica* (Milky Mushroom) (Subbiah and Balan, 2015; Vimala and Das, 2009)
- *Pleurotus ostreatus* (Oyster Perl Mushroom) (da Luz *et al.*, 2013; Qu *et al.*, 2015; Salame *et al.*, 2013; Singh and Singh, 2012; Skariyachan *et al.*, 2016; Younis *et al.*, 2015)

3. Preparation of spawn and cultivation of selected mushroom species will be grown by the protocols suggested in Cultivation Technology of Mushrooms (2007) NRCM (ICMR) Chambagaht, Solan (H.P)

- Mother culture will be further maintained on PDA (Potato Dextrose Agar) medium in petri-dishes
- Preparation of substrate for the growth of mushroom will be prepared

4. Isolation and identification of bacteria from Dayalbagh STP water will be performed by the step-wise suggested protocols given below:

- The bacterial isolates present in the contaminated STP sites will be isolated by Serial dilution (Pour-Plate) technique.
- The isolated bacterial cultures will be identified on the basis of their morphological, physiological and biochemical characteristics features by Bergey's Manual of Systematic Bacteriology.
- These cultures will be further cross examined by BD-BBL Crystal Identification Autoreader (Becton Dickinson and Company, USA) for the identification surety.

5. Antibacterial activity of mushroom against pathogenic bacteria procured from MTCC will be determined by the step-wise suggested protocol given below:

- Extract preparation from fruit bodies and mycelial culture will be prepared (Dong *et al.*, 2014; Mau *et al.*, 2001; Vamanu and Nita, 2013; Younis *et al.*, 2015)
- The antimicrobial assay will be performed by agar disc diffusion methods(Chowdhury *et al.*, 2015) (Cai *et al.*, 2015; Ramesh and Pattar, 2010; Younis *et al.*, 2015).
- Antimicrobial activities will be determined by measuring the diameter (in millimetre) of the zone of inhibition (M J Alves *et al.*, 2012; Maria José Alves *et al.*, 2012)

6. Remediation of heavy metals and pathogenic bacteria from STP water using mushroom in mycofiltration technique will be performed in step-wise protocol given below:

- Mycofiltration technique will be used following Paul Stamtes method

Chemical toxicity test of mushroom: A drop of juice was pressedout of the fresh fruit body on a piece of newspaper. After the spot had dried, hydrochloric acid was dropped on it. A blue spot indicated the presence of toxins (Feeney *et al.*, 2014; Ohnuki *et al.*, 2016).

SIGNIFICANCE OF THE WORK

- Using waste to grow mushrooms and remediate waste through mushrooms is the best approach of green technology of mycoremediation.
- Antimicrobial activity against various microbes present in contaminated sites increase the survivability of mushrooms using for bioremediations & decreases the population of harmful microbes.
- Mushrooms are easily grown and accumulate large amount of heavy metals, waste and other toxic chemicals. They can convert complex compounds into simple degradable compounds.
- Mycofiltration can facilitate reuse of wastewater in agricultural, botanical park irrigation purposes other examples in which it can contribute remarkable work are: Life stock farms in violation, Individual landowners of leaking septic tank violations, “Buffers” or “riparian buffers” in National Forests where endangered or key-stone species live, Native Tribal lands, Commercial Fishing Industries, State Agencies, Watershed buffers, Community garden buffers, Individual landowners with adjacent neighbors who create pollution that travels onto their properties, Housing developments with stormwater regulations or violations, Landscape companies, Horse boarders, horse pastures, Agricultural runoff (cow, pig, etc.), Municipalities, Conservation organizations, Restoration organizations, Land-use developers etc,.
- Mushrooms can also be utilized as bioreactors in industry for the synthesis of proteins and pharmaceutical compounds (Tang et al., 2007).
- A higher biomass of mushrooms can be produced on low-cost waste materials in a secure containment facility with the option of automation and mechanical harvesting (Lee et al., 2002).
- The proteins manufactured in mushrooms will also have higher specific biological activities in humans than those produced from plants (Lum and Min, 2011).

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